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## Therapeutic potential of KCa3.1 blockers: an overview of recent advances, and promising trends

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### Abstract

The calcium-activated potassium channel  $K_{Ca3.1}$  regulates membrane potential and calcium signaling in erythrocytes, activated T and B cells, macrophages, microglia, vascular endothelium, epithelia, and proliferating vascular smooth muscle cells and fibroblasts.  $K_{Ca3.1}$  has therefore been suggested as a potential therapeutic target for diseases such as sickle cell anemia, asthma, coronary restenosis after angioplasty, atherosclerosis, kidney fibrosis and autoimmunity, where activation and excessive proliferation of one or more of these cell types is involved in the pathology. This article will review  $K_{Ca3.1}$ 's physiology and pharmacology and critically examine the available preclinical and clinical data validating  $K_{Ca3.1}$  as a therapeutic target.

### Keywords

***K<sub>Ca3.1</sub>***: intermediate-conductance calcium-activated  $K^+$  channels (also known as IK1, SK4 or KCNN4); ***Sickle-cell disease***: is a life-long blood disorder characterized by red blood cells that assume an abnormal, rigid, sickle shape because of a mutation in the hemoglobin gene. "Sickling" decreases the flexibility of the erythrocytes and results in a risk of various complications such as baseline anemia, vaso-occlusive events and stroke.; ***ICA-17043***: 4-fluoro- $\alpha$ -(4-fluorophenyl)- $\alpha$ -phenyl-benzeneacetamide,  $K_{Ca3.1}$  blocker that entered clinical trials as Senicapoc®; ***TRAM-34***: 1-[(2-chlorophenyl)diphenylmethyl]-1*H*-pyrazole, commonly used experimental  $K_{Ca3.1}$  blocker; ***Atherosclerosis***: is a disease of large and medium-sized muscular arteries and is characterized by endothelial dysfunction, vascular inflammation, and the buildup of lipids, cholesterol, calcium, and cellular debris within the intima of the vessel wall.; ***Restenosis***: means the reoccurrence of stenosis, a narrowing of a blood vessel, leading to restricted blood flow. It typically occurs following angioplasty and is caused by the proliferation of intimal vascular smooth muscle cells (neointimal hyperplasia); ***Fibrosis***: is the formation or development of excess fibrous connective tissue in an organ or tissue.; ***Asthma***: common chronic disorder of the airways characterized by variable and recurring symptoms, airflow obstruction, smooth muscle hypertrophy, bronchial hyperresponsiveness (bronchospasm), and an underlying inflammation.

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## Expert Commentary

### Introduction/Background

Calcium-activated K<sup>+</sup> (K<sub>Ca</sub>) channels open in response to increases in cytosolic calcium and play important roles in modulating calcium-signaling and membrane potential in both excitable and non-excitable cells. The human genome contains eight K<sub>Ca</sub> channels, which can be divided into two groups depending on their genetic relationship, their single channel conductance and the molecular mechanism of their calcium-“sensing” [1]. The first group encompasses K<sub>Ca</sub>1.1, K<sub>Ca</sub>4.1, K<sub>Ca</sub>4.2 and K<sub>Ca</sub>5.1, which were initially grouped together based on structural similarity but later found to differ in their activation mode. While the founding member of the group, K<sub>Ca</sub>1.1 (BK), is indeed a “true” K channel and is activated by Ca<sup>2+</sup> Ca binding to a negatively charged segment in the C-terminus, K<sub>Ca</sub>4 and K<sub>Ca</sub>5 channels are activated by sodium, chloride or alkalization [1]. The channels were initially grouped together based on their structural similarity. The second group consists of the three small-conductance channels K<sub>Ca</sub>2.1 (SK1), K<sub>Ca</sub>2.2 (SK2), and K<sub>Ca</sub>2.3 (SK3), and the intermediate-conductance channel K<sub>Ca</sub>3.1 (IK1, SK4), which is the focus of this review (Figure 1A). K<sub>Ca</sub>3.1 was cloned by three groups in 1997 [2-4] and found to show about 42-44% sequence identity to the K<sub>Ca</sub>2 channels. Like the K<sub>Ca</sub>2 channels, K<sub>Ca</sub>3.1 is voltage-independent and is activated with reported EC<sub>50</sub>s of 95 to 350 nM by intracellular calcium binding to the EF-hands of calmodulin (Figure 1B), which is constitutively associated with the C-terminus of the channel and serves as its calcium-sensing β-subunit [5,6]. At the gene level, K<sub>Ca</sub>3.1 transcription can be repressed by the repressor element-1 silencing transcription factor (REST) [7] and increased through the transcription factors AP-1 (activation protein-1) and Ikaros-2 [8]. At the protein level, K<sub>Ca</sub>3.1 function is increased by protein kinase A (PKA) [9] and nucleoside diphosphate kinase B (NDPK-B) [10] and inhibited by the histidine phosphatase PHPT1 [11]. NDPK-B and PHPT1 directly phosphorylate/dephosphorylate K<sub>Ca</sub>3.1 on histidine 358 in the C-terminus (Figure 1B) and K<sub>Ca</sub>3.1 modulation thus constitutes one of the rare examples of a histidine kinase/phosphatase regulating a biological process in mammals.

Although so far no human diseases involving K<sub>Ca</sub>3.1 mutations have been described, K<sub>Ca</sub>3.1 constitutes a very attractive and (in some cases) relatively well-validated drug target for diseases or conditions ranging from sickle cell disease, restenosis and atherosclerosis to asthma and traumatic brain injury. In this article we will briefly summarize the physiological and pathophysiological role of K<sub>Ca</sub>3.1, review the existing pharmacological tool compounds and drug candidates, and then discuss the future perspectives of K<sub>Ca</sub>3.1 as a therapeutic target.

### Physiological role of K<sub>Ca</sub>3.1

In mammals, K<sub>Ca</sub>3.1 channels are widely expressed throughout the body but are absent from excitable tissues such as cardiac myocytes, skeletal muscle and the nervous system [2-4]. The only exceptions seem to be certain enteric neurons, where K<sub>Ca</sub>3.1 has been suggested to underlie part of the apamin-insensitive slow after hyperpolarization [12]. K<sub>Ca</sub>3.1 channels are primarily found in hematopoietic derived cells (i.e erythrocytes, platelets, lymphocytes, mast cells, monocytes/macrophages); epithelial tissues in the gastrointestinal tract, lung and endo- and exocrine glands; as well as vascular endothelial cells, fibroblasts and proliferating neointimal vascular smooth muscle cells [3,4,8,13-17].

Our initial understanding of the physiological role of K<sub>Ca</sub>3.1 came from studies in the late 1950's when Gárdos demonstrated that potassium efflux in erythrocytes was activated by calcium entry [18]. Later studies confirmed that the underlying potassium conductance (called the “Gárdos channel”) is K<sub>Ca</sub>3.1 [19,20] and that efflux of potassium ions through

this channel is accompanied by a loss of cellular water leading to dehydration and erythrocyte shrinkage. Potassium efflux through  $K_{Ca3.1}$  can indeed be quite large as application of calcium ionophores can result in the efflux of greater than 50% of the cell's intracellular potassium content. In some situations this cell shrinkage is associated with programmed cell death (apoptosis) [21]. In addition to erythrocytes,  $K_{Ca3.1}$  has also been reported to be involved in lymphocyte apoptosis by mediating cell shrinkage in calcium-induced apoptosis or P2X<sub>7</sub>-receptor stimulated cell death in lymphocytes [22,23]. However, it is currently not clear whether the recently reported  $K_{Ca3.1}$  expression in the inner mitochondrial membrane is involved in these processes [24]. A similar involvement in volume regulation of other hematopoietic cell types has also been demonstrated. For example, in transgenic mice where  $K_{Ca3.1}$  is functionally absent, both T-lymphocytes and mast cells exhibit attenuated responses to environmental osmotic changes [19,20].

Perhaps the most well defined role of  $K_{Ca3.1}$  channels is to regulate calcium entry into cells and thereby modulate calcium-signaling processes. The channel performs this duty by helping to control membrane potential (Figure 2). Entry of positively charged calcium depolarizes the membrane, which limits its own ability to enter the cell through inward-rectifier-type calcium channels like CRAC (calcium release activated calcium channel) or some transient receptor potential channels, which are closed at more positive membrane potentials. Activation of  $K_{Ca3.1}$  by elevated intracellular calcium maintains a negative membrane potential, which helps to sustain calcium entry into the cell.  $K_{Ca3.1}$ -mediated elevation of intracellular calcium is necessary for the production of inflammatory chemokines and cytokines by T cells, macrophages and mast cells [8,25]. It is also a prerequisite for the proliferation of many cell types. Indeed, following stimulation of cells with growth factors (in the case of fibroblast or vascular endothelial and smooth muscle cells) or antigens or mitogens (in the case of T and B cells) proliferation is accompanied by transcriptional up-regulation of functional  $K_{Ca3.1}$  expression and can be inhibited by  $K_{Ca3.1}$  blockers [8,15,26,27].

$K_{Ca3.1}$  mediated control of calcium entry has further been shown to be involved in the migration of macrophages [28], microglia [29], vascular smooth muscle cells [30,31], and mast cells [25]. In microglia  $K_{Ca3.1}$  also seems to play a role in the oxidative burst, nitric oxide production and microglia-mediated neuronal killing [32,33]. There is also evidence that activation of  $K_{Ca3.1}$  may improve in platelet coagulation [34].

Epithelial cells are the major conduit for electrolyte and fluid transport in tissues and organs throughout the body.  $K_{Ca3.1}$  channel protein is expressed in the epithelium lining the gastrointestinal tract, in lung epithelia, in ducts of fluid secreting glands (i.e. salivary, lacrimal, pancreas, prostate) as well as in stratified epithelia, including skin, the cornea, oral mucosa and urothelium [35-38]. In addition to serving a protective role in cells undergoing osmotic challenge,  $K_{Ca3.1}$  channels, which are typically expressed on the basolateral membrane, provide a polarized pathway for potassium flux (alone or in partnership with  $K_{V7.1}$  channels), which helps to facilitate chloride secretion and consequently water transport across the epithelia [35,37].

Together with the small-conductance  $K_{Ca2.3}$  channel,  $K_{Ca3.1}$  initiates the so-called endothelium derived hyperpolarization factor (EDHF) response in vascular endothelium, which causes subsequent relaxation of the underlying vascular smooth muscle cell layer through hyperpolarization mediated via closure of voltage-gated calcium channels [39-41]. As expected, genetic deficiency of both  $K_{Ca3.1}$  and  $K_{Ca2.3}$  largely abolish EDHF-type dilator responses and increases mean blood pressure in double transgenic animals [42]. However, the single transgenics revealed that the two endothelial  $K_{Ca}$  channels have distinct stimulus dependent functions. While  $K_{Ca3.1}$  deficiency severely impairs acetylcholine-

mediated vasodilations [43],  $K_{Ca2.3}$  deficiency significantly reduces sheer-stress stimulated vasodilations [42]. These distinct functions seem to be related to a spatial separation of the two  $K_{Ca}$  channels within the vascular endothelium.  $K_{Ca2.3}$  is found at endothelial cell junctions [44], whereas  $K_{Ca3.1}$  is localized to the endothelial projections through the holes in the elastic lamina, which are also the sites of the myoendothelial gap junctions that electrically couple endothelial cells to the underlying vascular smooth muscle cells. In these projections,  $K_{Ca3.1}$  is seen in close proximity to the endoplasmic reticulum and has been proposed to be preferentially activated by acetylcholine-triggered “calcium pulsars” through calcium release from the endoplasmic reticulum [45].

It is also worth noting here that despite the wide expression of  $K_{Ca3.1}$ , the functional absence of this channel in transgenic knockout mice has not been associated with gross physiological changes. Animals constitutively lacking  $K_{Ca3.1}$  are of normal appearance; reproduce normally and have no abnormalities of any major organs. Apart from impairments in cell volume regulation in erythrocytes and T lymphocytes [20], subtle erythrocyte macrocytosis and progressive splenomegaly during aging [46], a mild 7 mmHg increase in mean arterial blood pressure associated with the reduced EDHF response [42,43],  $K_{Ca3.1}^{-/-}$  mice exhibit relatively normal phenotypes. A recent study on  $K_{Ca3.1}^{-/-}$  mice has further reported that their pancreatic beta-cells exhibited increased calcium action potential frequencies and accordingly secreted more insulin at lower glucose concentrations [47]. The mice were not hypoglycemic but showed improved glucose tolerance, which raises the possibility that  $K_{Ca3.1}$  blockers might constitute novel insulinotropic drugs [47]. It should of course be noted that all these findings are of limited significance because of the possibility of developmental compensations in constitutive knock-out mice.

### Available Drugs and Tool compounds

In contrast to many other potassium channels,  $K_{Ca3.1}$  has a relatively well-developed pharmacology, which was recently reviewed by us in great detail and put into its historical perspective [48]. We will therefore here only briefly describe the most commonly used  $K_{Ca3.1}$  modulators and then concentrate our discussion on available animal model or clinical trial data.

Traditionally  $K_{Ca3.1}$  had been distinguished from the apamin-sensitive  $K_{Ca2}$  channels by its sensitivity to the scorpion toxin charybdotoxin (ChTX). However, ChTX ( $IC_{50}$  5 nM) was never an ideal  $K_{Ca3.1}$  blocker because it also inhibits  $K_{V1.3}$  and  $K_{Ca1.1}$ , which made it problematic to use on any cell type that expresses these channels. Another scorpion toxin that potently blocks  $K_{Ca3.1}$  is maurotoxin (MTX,  $IC_{50}$  1 nM). However, MTX has an even higher affinity ( $IC_{50}$  100 pM) for  $K_{V1.2}$  and attempts by scientists at Icagen in collaboration with the laboratory of Jean-Marc Sabatier to design  $K_{Ca3.1}$ -selective derivatives remained unsuccessful [49,50]. Similar efforts by the group of George Chandy produced the ChTX analog ChTX-Glu<sup>32</sup>, whose negatively charged Glu<sup>32</sup> is repelled by negatively charged residues in the outer vestibule of  $K_{V1.3}$ , resulting in 30-fold selectivity for  $K_{Ca3.1}$  over  $K_{V1.3}$  [51]. However, this modification did not increase selectivity over  $K_{Ca1.1}$ .

In addition to peptide toxins,  $K_{Ca3.1}$  is inhibited by a number of “old drugs” including the antimalarial quinine ( $IC_{50}$  2+ 50 100  $\mu$ M), the vasodilator cetiedil ( $IC_{50}$  25  $\mu$ M), the L-type Ca channel blockers nifedipine ( $IC_{50}$  4  $\mu$ M) and nitrendipine ( $IC_{50}$  1  $\mu$ M), and the antimycotic clotrimazole (**1**,  $IC_{50}$  70-250 nM) [48]. Interestingly, clotrimazole (**1**) was initially postulated to affect  $K_{Ca3.1}$  through a P450-like heme protein, when it was first reported to block calcium-activated potassium efflux in erythrocytes by Alvares et al. in 1992 [52]. This hypothesis was later proven to be incorrect when Brugnara and coworkers showed that clotrimazole’s metabolite (2-chlorophenyl)diphenylmethanol, which lacks the imidazole ring required for P450 inhibition, also blocked the Gárdos channel [53]. Brugnara

and his coworkers at the Children's Hospital at Harvard University also quickly recognized the therapeutic implications of Alvarez' observation and used clotrimazole (**1**) as a tool to demonstrate that pharmacological  $K_{Ca3.1}$  blockade reduced erythrocyte dehydration in both a mouse model of sickle-cell anemia [54] and a small number of patients with the disease [55]. However, clotrimazole (**1**) itself was not suitable for long-term use in patients or experimental animals because of its acute inhibition and chronic induction of human cytochrome P450-dependent enzymes [56,57] leading to liver damage and changes in cortisol levels. Three groups therefore used clotrimazole (**1**) as a template for the design of triarylmethane based  $K_{Ca3.1}$  blockers, which are free of cytochrome P450 inhibition [48]. Brugnara and colleagues synthesized a number of triarylmethanes and patented them for sickle cell disease and abnormal cell proliferation [201]. By replacing clotrimazole's imidazole ring systematically with other heteroaromatic ring systems or various functional groups, Wulff et al. identified TRAM-34 (**2**), a compound that inhibits  $K_{Ca3.1}$  with an  $IC_{50}$  of 20 nM, has no effect on the P450 enzyme CYP3A4, and exhibits 200-500 fold selectivity over  $K_V$  channels and 1000-fold selectivity over  $K_{Ca1.1}$  and  $K_{Ca2}$  channels [58]. Through site-directed mutagenesis it was later demonstrated that TRAM-34 (**2**) and clotrimazole (**1**) interact with Thr<sup>250</sup> in the pore loop and Val<sup>275</sup> in S6 and therefore constitute "inner pore" blockers [59]. Independently of the two other groups, scientists at Icagen Inc. explored fluorinated triphenyl acetamides [60]. The compound with the best pharmacokinetic properties from this series is ICA-17043 (**3**, Senicapoc®), which inhibits  $K_{Ca3.1}$  with an  $IC_{50}$  of 11 nM and is orally bioavailable in humans with a half-life of 12.8 days [61]. ICA-17043's greatly increased metabolic stability in comparison to clotrimazole (**1**) and TRAM-34 (**2**), which has a half-life ~2 hours in rhesus macaques and is acid sensitive and not orally available despite attempts at microencapsulation [62], is probably due to the presence of the two fluorine substituents in *para* position on two of the phenyl rings, which seem to greatly reduce oxidative metabolism [60]. More recently reported "triarylmethane-like"  $K_{Ca3.1}$  blockers are 11-phenyl-diazepines exemplified by compound **4** ( $IC_{50}$  for Gárdos channel blockade ~90 nM) and diphenylindanones exemplified by **5** ( $IC_{50}$  189 nM), which were patented by Brugnara and co-workers [202,203]. Aromatically substituted sulfonamides, exemplified by **6** (*N*-(4-methyl-2-oxazol-2yl-phenyl)-3-trifluoromethyl-benzenesulfonamide) are a more structurally distantly related class of  $K_{Ca3.1}$  blockers, described in an Icagen Inc. patent [204], which inhibit <sup>86</sup>Rb flux through Gárdos channels in human erythrocytes with  $IC_{50s}$  lower than 500 nM.

Another class of  $K_{Ca3.1}$  blockers, which was first reported on in literature in 2003, are the 4-phenyl-4*H*-pyrans [63] and the related cyclohexadienes [64]. While initial attempts by chemists at Neurosearch A/S to use the dihydropyridine nifedipine (**7**) as a template for the design of a potent  $K_{Ca3.1}$  blocker were unsuccessful, Urbahns et al. at Bayer reasoned that isoelectronic replacement of the NH required for voltage-gated calcium channel blockade with an O might lead to more potent and specific  $K_{Ca3.1}$  blockers [63]. This assumption turned out to be accurate since even a simple dimethyl ester substituted 4-phenyl pyran blocked  $K_{Ca3.1}$  with an affinity of 160 nM. This activity was then further enhanced through additional derivatization on the phenyl ring and finally resulted in a 4-Cl, 3-CF<sub>3</sub> substituted phenyl-4*H*-pyran (**8**) that blocked  $K_{Ca3.1}$  with an  $IC_{50}$  of 8 nM. A subsequent exchange of the NH with a CH<sub>2</sub> group through a Carba-Hantzsch reaction resulted in the cyclohexadienes [64] exemplified by compound **9** ( $IC_{50}$  of 1.5 nM). However, the cyclohexadienes showed a tendency to 3,6/3,5 double bond isomerization and the authors therefore also synthesized cyclohexadiene lactones, in which the second ring prevents isomerization. The exemplary cyclohexadiene lactone **10** inhibits  $K_{Ca3.1}$  with an  $IC_{50}$  of 8 nM and exhibits a good selectivity over other potassium, calcium and sodium channels as well as transporters and receptors in binding assays [65]. Tissue distribution studies with **9** further showed that the compound is 10-fold enriched in brain tissue [64] suggesting that it

might be ideal for studying the role of  $K_{Ca3.1}$  in indications that require penetration into the brain. Interestingly, the title of the German patent [205] claiming the initial compounds in 1997 was incorrectly translated into English as “Preparation of dimethyl-substituted cyclohexadiene-derivative calcium channel modulators” which might explain why many companies outside of Germany were initially not aware of the existence of these compounds.

In addition to the above-described blockers, scientists studying  $K_{Ca3.1}$  also have the possibility to pharmacologically increase  $K_{Ca3.1}$  activity with a number of small molecule activators, which increase the single channel open probability in a calcium-dependent manner resulting in an apparent leftward shift of the calcium activation curve by an order of magnitude [66-68]. However, care should be taken not to term these compounds “openers” because they are ineffective in the absence of calcium and therefore do not “open” the channel per se. The “classical”  $K_{Ca3.1}$  activator is ethylbenzimidazolone (EBIO, **11**), which was first described by Devor et al. in 1996 [36] and later confirmed to activate cloned  $K_{Ca3.1}$  channels with an  $EC_{50}$  of 30  $\mu$ M. More potent  $K_{Ca3.1}$  activators include 5,6-dichloro-EBIO (**12**, DCEBIO,  $EC_{50}$  1  $\mu$ M) [69], the Neurosearch AS compound NS309 (**13**,  $EC_{50}$  30 nM) [70], the neuroprotectant riluzole (**14**,  $EC_{50}$  2  $\mu$ M) and its recently identified more selective derivative SKA-31 (**15**,  $EC_{50}$  250 nM) [71]. However, all these compounds are not perfectly selective for  $K_{Ca3.1}$  and only display a 3 to 5-fold selectivity for  $K_{Ca3.1}$  over the  $K_{Ca2}$  channels. DC-EBIO and NS309 also block L-type calcium channels [72] and in the case of NS309 the cardiac hERG (Kv11.1) channel and are therefore not suitable for human use. The only  $K_{Ca3.1}$  activator that has been demonstrated so far to be useful *in vivo* is SKA-31 (**12**), which has surprisingly good pharmacokinetics with low plasma protein binding and a plasma half-life of 12 hours in rats [71]. SKA-31 (**15**) has recently been shown to potentiate EDHF-type dilations in murine carotid arteries and to lower mean arterial blood pressure in both normotensive and hypertensive mice in a  $K_{Ca3.1}$ -dependent fashion, suggesting  $K_{Ca3.1}$  channel activation as a potential new strategy for the treatment of hypertension [42,71]. However, compared with the body of knowledge discussed below for  $K_{Ca3.1}$  blockers, this indication for  $K_{Ca3.1}$  activators needs to be validated further since it is currently not clear whether  $K_{Ca3.1}$  activators will be effective in more long-term experiments. It further remains to be seen what the side-effect profile of  $K_{Ca3.1}$  activators will be. Based on the expression of  $K_{Ca3.1}$  in various prostate, breast and pancreatic cancer cell lines [73]  $K_{Ca3.1}$  activators could potentially induce neoplastic changes.

### Pathophysiological role of $K_{Ca3.1}$

In developing an understanding of the physiological role of  $K_{Ca3.1}$ , it has become evident that this channel may also play an important role in various pathophysiological disorders. First, aberrant dehydration of red blood cells in people with hereditary sickle cell disease can precipitate polymerization of mutated hemoglobin S resulting in the sickling and eventual destruction of erythrocytes. The contribution of  $K_{Ca3.1}$  to the etiology of this disease is supported by the finding that inhibiting calcium dependent flux of potassium with the  $K_{Ca3.1}$  inhibitors clotrimazole (**1**) and Senicapoc® (**3**) results in a reduction in hemolysis as well as in reduced numbers of dense erythrocytes [54,74,75], which are a precursor to sickled cells. Second, while the cellular processes underlying immune responses to infection and/or tissue damage are typically well orchestrated, under certain conditions they can become unbalanced leading to a variety of autoimmune and inflammatory disorders. For example, in many inflammatory disorders, immune responses are over-amplified or lack a resolution process. As such, it is not uncommon for migration, activation and proliferation of immunologically active cells like lymphocytes, macrophages and mast cells to become uncontrolled leading to further tissue damage and disease progression. Activation of  $K_{Ca3.1}$  is believed to contribute to a number of these cellular events. For example,  $K_{Ca3.1}$

expression is up-regulated in activated naïve and central memory T-cells and IgD<sup>+</sup> B-cells [27,76] and the channel has therefore been proposed as a target for the treatment of autoimmune diseases and transplant rejection [77]. In animal models, the K<sub>Ca</sub>3.1 inhibitors TRAM-34 (2) or Senicapoc® (3) have been reported to prevent experimental autoimmune encephalomyelitis (EAE) induced by immunization with MOG peptide in mice [78] or to inhibit/attenuate inflammatory responses in mice with collagen antibody induced rheumatoid arthritis [79]. Indeed it is worth noting that the archetypal K<sub>Ca</sub>3.1 inhibitor clotrimazole (1) was reported to improve the symptoms of rheumatoid arthritis in a small clinical trial in the 1980s [80]. The combination of TRAM-34 (2) with the K<sub>v</sub>1.3 blocking peptide ShK was further recently shown to reduce T cell and macrophage infiltration in the early stages of chronic kidney transplant rejection in rats [81], suggesting that K<sub>Ca</sub>3.1 blockers should be further investigated in chronic transplant rejection models. The group of Edward Skolnik also recently demonstrated that TRAM-34 (2) significantly reduced the severity of TNBS induced colitis in mice, a model of human inflammatory bowel disease [82]. This finding was supported by the observation that adoptive transfer of T lymphocytes from K<sub>Ca</sub>3.1<sup>-/-</sup> mice failed to induce colitis in rag2<sup>-/-</sup> mice in contrast to wild type T cells.

K<sub>Ca</sub>3.1 channels have also been reported to play various roles in mast cell function. Early studies by Bradding and colleagues demonstrated functional expression of K<sub>Ca</sub>3.1 channels in human lung mast cells and showed that inhibition of this channel with agents like TRAM-34 (2) affected both degranulation and migration responses [25]. Furthermore, K<sub>Ca</sub>3.1 appears to contribute to the modulation of mast cell function through its regulation by a variety of receptors (i.e. beta adrenoceptor, adenosine and prostaglandin) known to play a role in asthma and other inflammatory airway disorders [83]. A potential role of K<sub>Ca</sub>3.1 in the pathophysiology of airway inflammatory diseases [84] is supported by the observation that the K<sub>Ca</sub>3.1 inhibitor Senicapoc® (3) reduces allergen challenge induced increases in airway resistance and hyper-reactivity in a sheep model of asthma [85]. K<sub>Ca</sub>3.1 may also participate in neuroinflammation. In cultured microglia K<sub>Ca</sub>3.1 is involved in the respiratory burst [32], iNOS induction, nitric oxide production [33], migration [29] and in microglia-mediated neuronal killing [33]. It should of course be noted that cultured microglia are not necessarily a good model of microglia *in vivo*. Intraocular injection of the K<sub>Ca</sub>3.1 inhibitor TRAM-34 (2) reduces retinal ganglion cell degeneration after optic nerve transection in rats [33]. Interestingly, K<sub>Ca</sub>3.1 blockade does not prevent microglia from phagocytosing damaged neurons but increases the number of surviving retinal ganglion cells, presumably by reducing the production and/or secretion of neurotoxic molecules in the retina [33]. Additional evidence for a neuroprotective role for K<sub>Ca</sub>3.1 blockers comes from the observation that both cyclohexadiene lactone and triaryl methane – type K<sub>Ca</sub>3.1 inhibitors reduce brain edema and infarct volume caused by traumatic brain injury in rats [65].

The contribution of K<sub>Ca</sub>3.1 to pathophysiological inflammatory and proliferative responses can be further highlighted by the role of the channel in vascular diseases, particularly in restenosis, atherosclerosis and kidney fibrosis. TRAM-34 (2) has been demonstrated to significantly reduce balloon angioplasty induced neointimal smooth muscle hyperplasia (restenosis) in the carotid artery in rats [15]. Similar observations have been made in a porcine coronary overstretch injury model, which very closely mimics post-angioplasty injury seen in humans. TRAM-34 (2) administered directly to the coronary vessel wall at the time of the balloon catheter angioplasty was found to significantly reduce the magnitude of restenosis observed 28 days post surgery [86]. Again using TRAM-34 (2), the group of Hiroto Miura showed that K<sub>Ca</sub>3.1 blockade significantly reduced atherosclerosis development in ApoE<sup>-/-</sup> mice by reducing smooth muscle cell proliferation and lymphocyte infiltration into atherosclerotic plaques [87]. However, in blood vessels K<sub>Ca</sub>3.1 does not only drive the proliferation of dedifferentiated smooth muscle cells but also the proliferation

of microvascular and macrovascular endothelial cells and  $K_{Ca}3.1$  blockade therefore also potently suppresses angiogenesis in the mouse matrigel plug assay [17].

$K_{Ca}3.1$  has further long been known to play a role in fibroblast proliferation [13] but the relationship of this action to pathophysiology was not clearly evident until a recent study reported that genetic disruption of  $K_{Ca}3.1$  or pharmacological blockade of  $K_{Ca}3.1$  with TRAM-34 (**2**) significantly reduces renal fibrosis in mice and rats following unilateral ureteral obstruction [88]. The contribution of  $K_{Ca}3.1$  to fibrosis in other tissue remains to be determined.

Given the extensive evidence that  $K_{Ca}3.1$  plays an important role in the proliferative responses of many cell types, it is perhaps not surprising that this channel has also been reported to contribute to the development of various forms of cancer.  $K_{Ca}3.1$  is involved in the proliferation of human LNCaP and PC-3 prostate cancer cells, leukemic HL-60 and glioblastoma GL-15 cells, MCF-7 breast cancer cells, BxPC-3 and MiaPaCa-2 pancreatic cancer cells, and HEC-1A and KLE endometrial cancer cells [see [73] for review]. Two *in vivo* studies provide some evidence for the hypothesis that  $K_{Ca}3.1$  could constitute a target for cancer treatment. Benzaquen et al. showed in 1995 that clotrimazole (**1**) reduced the number of lung metastases in SCID mice that were inoculated with human melanoma cells [89], while Zhang et al. demonstrated in 2007 that both clotrimazole (**1**) and TRAM-34 (**2**) slowed the growth of tumors in nude mice injected with human endometrial cancer cells [90]. Furthermore, due to the inhibition of angiogenesis by  $K_{Ca}3.1$  inhibitors, a role as an adjuvant in inhibition of tumor angiogenesis could be pursued [17].

The role of  $K_{Ca}3.1$  in epithelial tissue dysfunction remains to be fully explored. However, there are reports suggesting that  $K_{Ca}3.1$  channels are critical for cAMP-dependent chloride secretion and cyst growth in autosomal-dominant polycystic kidney disease [91]. Furthermore, an abolition of  $Ca^{2+}$ -mediated intestinal anion secretion and increased fecal dehydration has been observed in transgenic mice lacking functional  $K_{Ca}3.1$  channels [92]. The observations are in line with reports from 1997 by Rufo et al. that clotrimazole (**1**) reduces chloride secretion *in vitro* by T84 cell monolayers and *in vivo* in mice that were challenged with cholera toxin to induce secretory diarrhea [93].

### Clinical experience with $K_{Ca}3.1$ blockers

Given its broad expression profile, as well as the extensive evidence supporting a role for  $K_{Ca}3.1$  in various pathophysiological mechanisms, development of modulators of this channel would appear to be an obvious strategy for pharmaceutical companies interested in developing treatments for one or more of diseases/disorders described above. The first reported drug development program focused on  $K_{Ca}3.1$  was for the treatment of sickle cell disease. The evidence for a role of  $K_{Ca}3.1$  in erythrocyte water homeostasis and viability in sickle cell disease is compelling [54,74]. An early attempt to target the disease using the weakly selective  $K_{Ca}3.1$  inhibitor clotrimazole (**1**) showed promise in a small clinical trial, although toxicity issues prevented further development [55]. Icagen Inc. followed these studies and targeted sickle cell disease for the clinical development of its more potent and selective  $K_{Ca}3.1$  blocker Senicapoc® (see [94] for a review). Senicapoc® proved to be very efficacious in an animal model of sickle cell disease [75], particularly reversing hemolysis and reducing the number of dense red blood cells, the precursor to sickled cells. In a randomized, double-blind, placebo-controlled phase I trial in healthy volunteers, Senicapoc® (**3**) was found to be safe and well tolerated at doses that effectively blocked a large percentage of Gárdos channels in the test subjects [61]. A 12-week, multi-center, randomized double-blind Phase II clinical trial showed that Senicapoc® (**3**) was able to reduce hemolysis and increase hemoglobin levels in sickle cell patients [95]. A subsequent Phase III study was designed to compare the rate of acute vaso-occlusive pain crises



occurring in sickle cell disease patients receiving 10 mg of Senicapoc® (3) daily for 48 weeks versus placebo. While Senicapoc® (3) reduced hemolysis and increased hemoglobin levels as seen in the Phase II trial [96], the study was terminated early due to a low probability of achieving a reduction in the primary clinical end point, the rate of vaso-occlusive pain crisis. Thus, despite achieving the desired biological endpoints, treatment did not reduce clinical painful crises.

While the results from the sickle cell disease clinical trials were disappointing, it was clear from these studies that Senicapoc® (3) was a safe and well tolerated drug candidate in humans, and that it was biologically active at the doses given. Therefore, Icagen Inc. explored other therapeutic opportunities for this drug candidate. Consistent with its ability to reduce the increase in airway resistance and hyper-reactivity in a sheep allergen challenge model of asthma [85] Senicapoc® (3) demonstrated encouraging results in a small phase II allergen challenge study in patients with allergic asthma. In this trial, following two weeks of Senicapoc® treatment at 40 mg/day there was a reduction in the late allergen mediated increase in airway resistance along with a reduction in the inflammatory marker exhaled nitric oxide. In contrast, in a second proof-of-concept Phase II trial, in which Senicapoc®'s effect on exercise induced asthma was examined, no improvement in lung function was observed following 4 weeks of treatment (40 mg/day p.o.) [101].

### Five-Year View

Initial clinical trials with the selective  $K_{Ca}3.1$  inhibitor Senicapoc® (3) have provided evidence that blocking  $K_{Ca}3.1$  produces biological effects in humans and is well tolerated. While the clinical benefits of  $K_{Ca}3.1$  inhibition have not been established to date, there is sufficient scientific rationale to expect that this channel will continue to be pursued as a drug development target by pharmaceutical companies.  $K_{Ca}3.1$  also constitutes an attractive target for autoimmune diseases such as rheumatoid arthritis and inflammatory bowel disease as well as for conditions like kidney fibrosis, post-angioplasty restenosis and atherosclerosis, which are associated with the proliferation of fibroblasts and dedifferentiated vascular smooth muscle cells. Of particular interest are atherosclerosis and restenosis given that 1) existing studies demonstrate that  $K_{Ca}3.1$  blockers work in multiple species (mice, rats, and pigs), 2) the channel is strongly expressed in the intimal and medial layers of blood vessels from patients with coronary artery disease [87], and 3) both conditions have an inflammatory component. Whether as oral agents or in conjunction with devices such as drug eluting stents,  $K_{Ca}3.1$  blockers may be particularly helpful in these important cardiovascular diseases. Recent studies on the beneficial effects of both genetic deletion and pharmacological  $K_{Ca}3.1$  blockade in inflammatory bowel disease [82] and kidney fibrosis [88] make these indications of interest as well. We may expect to see groups seeking to further validate such an approach given the need for new therapies for these disorders as well as for liver and lung fibrosis, the later of which plays a significant role in disease mediated airway remodeling [84].

Another intriguing area is the targeting of  $K_{Ca}3.1$  to reduce microglia activity in traumatic brain injury [65] and possibly ischemic stroke and other neurodegenerative disorders with an inflammatory component. Pharmaceutical companies of course tend to shy away from stroke as an indication since the failure of numerous drug development programs including the  $K_{Ca}1.1$  channel activator BMS-204352 [97] in Phase III clinical trials for this indication in 2000 [98]. However, we would like to point out here that microglia activation in the wake of an ischemic stroke or traumatic brain injury develops slowly and has been shown to continue up to the chronic cystic stages months after stroke in human brain [99]. Treatments aimed at neuroinflammation might therefore still be able to demonstrate beneficial effects if started hours or even days after an ischemic stroke or brain injury has occurred by reducing

inflammatory damage in the so-called penumbra around the necrotic core area of the infarct. Since the contribution of microglia to the pathology of stroke and Alzheimer's disease is currently a very active research area, we expect that  $K_{Ca}3.1$  modulators will be further evaluated for their ability to reduce detrimental microglia functions and that a clearer picture of their usefulness for CNS indications will emerge in the next 5-10 years.

Although pharmacological blockade of  $K_{Ca}3.1$  appears to be safe and well tolerated in animals and humans, the fact that genetic deletion of the channel impairs the EDHF response and raises mean arterial blood pressure in the transgenic animals by ~7 mmHg [42,43], raises concerns that  $KCa3.1$  blockers might increase blood pressure. However, blood pressure increases with  $K_{Ca}3.1$  blocker have so far not been observed in mice receiving TRAM-34 [87] or over 500 human volunteers and patients taking Senicapoc® (3) for up to two years [95], presumably because combined blockade of endothelial  $K_{Ca}3.1$  and  $K_{Ca}2.3$  is necessary to abolish the EDHF response. Taken together,  $K_{Ca}3.1$  constitutes an attractive, apparently safe and drugable target for indications ranging from atherosclerosis and fibrosis to inflammatory bowel disease and neuroinflammation. It is anticipated that discovery and development activities around this exciting target will increase in the next 5 years.

## Bibliography

\* = of interest

\*\* = of considerable interest

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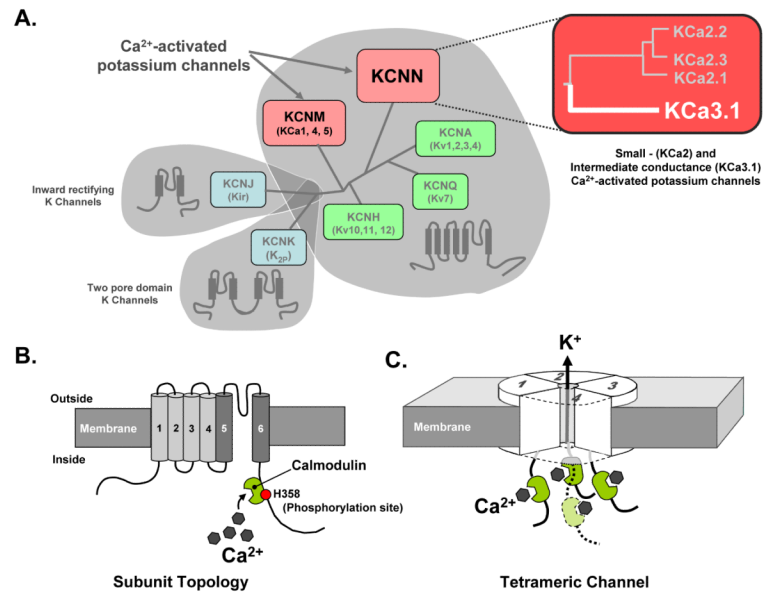
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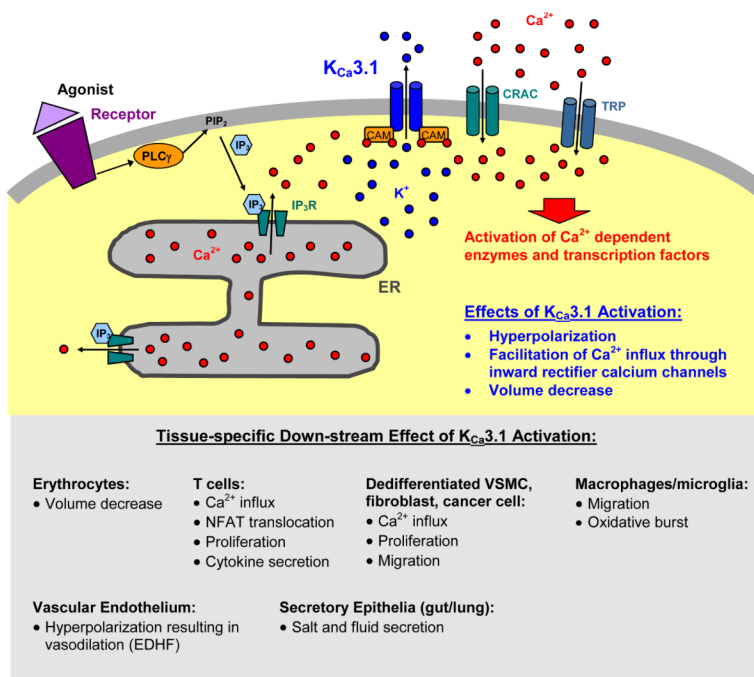
### Key Issues

- ◆  $K_{Ca}3.1$  regulates calcium-influx and membrane potential in erythrocytes, activated T and B cells, macrophages, mast cells, microglia, vascular endothelium, epithelia, and proliferating vascular smooth muscle cells and fibroblasts.
- ◆  $K_{Ca}3.1$  blockers inhibit immune cell migration and cytokine production and are effective in rodent models of EAE, IBD, rheumatoid arthritis and in an asthma model in sheep
- ◆  $K_{Ca}3.1$  blockers inhibit vascular smooth muscle cell proliferation and migration and reduce restenosis in rats and pigs
- ◆  $K_{Ca}3.1$  blockers prevent atherosclerosis development in ApoE<sup>-/-</sup> mice
- ◆  $KCa3.1$  blockers inhibit fibroblast proliferation in vitro and prevent kidney fibrosis in mice and rats
- ◆  $K_{Ca}3.1$  blockers decrease hemolysis and increase hemoglobin levels in patients with sickle cell disease but have not been shown to reduce the frequency vaso-occlusive crises in the clinic.
- ◆ The  $K_{Ca}3.1$  inhibitor Senicapoc® has been shown to reduce allergen induced increase in airway resistance in allergic asthma patients, but not the asthmatic responses to exercise challenge in patients with mild asthma.
- ◆ Despite the recent disappointing clinical trial results with  $K_{Ca}3.1$  modulators,  $K_{Ca}3.1$  remains a promising drug target for a variety of indications including restenosis, atherosclerosis, autoimmune diseases and possibly neuroinflammation.



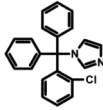
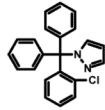
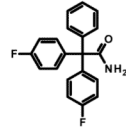
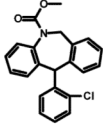
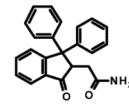
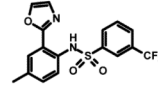
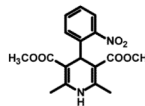
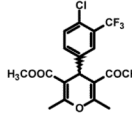
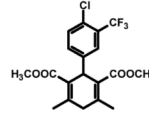
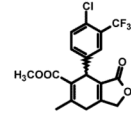
**Figure 1.**

Properties of  $K_{Ca3.1}$  channels. **A.** Simplified phylogenetic tree of genes for human potassium channel subunits, highlighting in red the two gene families that comprise  $Ca^{2+}$ -activated potassium channels and further highlighting the KCNN family which contains the gene for  $KCa3.1$  (KCNN4). **B.** Illustration of  $K_{Ca3.1}$  subunit topology showing the six transmembrane domain signature of this class of channels, along with calmodulin attached to a calmodulin binding domain on the C terminus. The location of the histidine phosphorylation site (H358) known to affect channel activation is also shown. **C.** Illustration of the homo-tetrameric nature of functional  $K_{Ca3.1}$  channels, showing the presence of four calmodulin calcium sensors which accounts for the channel's steep, highly cooperative sensitivity to changes in intracellular calcium concentration.

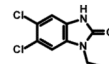
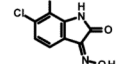
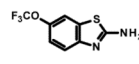
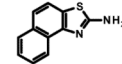


**Figure 2.** Cartoon of the physiological role of  $K_{Ca3.1}$ . The channel is activated by increases in intracellular calcium following calcium release from the ER (endoplasmic reticulum), and/or calcium influx through inward-rectifier calcium channels like CRAC (calcium release activated calcium channel) or TRP (transient receptor potential) channels. PLC; phospholipase C; IP<sub>3</sub> inositol-triphosphate; CAM; calmodulin.

## KCa3.1 Blockers

Clotrimazole (1)  
IC<sub>50</sub> 70-250 nMTRAM-34 (2)  
IC<sub>50</sub> 20 nMICA-17043 (3)  
IC<sub>50</sub> 11 nM11-Phenyl-dibenzazepine (4)  
IC<sub>50</sub> 90 nMDiphenylindanone 12 (5)  
IC<sub>50</sub> 189 nMSulfonamide 77 (6)  
IC<sub>50</sub> < 0.5 μMNifedipine (7)  
IC<sub>50</sub> 4 μM4-Phenyl-4H-pyran (8)  
IC<sub>50</sub> 8 nMCyclohexadiene 4 (9)  
IC<sub>50</sub> 1.5 nMCyclohexadiene Lactone (10)  
IC<sub>50</sub> 8 nM

## KCa3.1 Activators

EBIO (11)  
EC<sub>50</sub> 30 μMDCEBIO (12)  
EC<sub>50</sub> 1 μMNS309 (13)  
EC<sub>50</sub> 30 nMRiluzole (14)  
EC<sub>50</sub> 2 μMSKA-31 (15)  
EC<sub>50</sub> 250 nM

**Figure 3.**  
Structures of K<sub>Ca</sub>3.1 inhibitors and activators.